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Studies on the metabolism and excretion of chlorine chloride : a thesis ...

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STUDIES ON THE METABOLISM AND EXCRETION OF CHOLINE CHLORIDE

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INTRODUCTION

I. Historical

The organic base choline is of considerable interest to the physiologist because of its role in normal metabolic function, and to the pharmacologist because of its possible application in the treatment of several human diseases.

Although choline has been recognized as a chemical since its discovery in hog bile in 1862 (4), many years passed before its importance in metabolism was established. In 1934, Toda (23) found that oral administration of choline results in increased amounts of trimethylamine in the urine, but no choline excretion. He theorized that this is an enzymatic process because significant amounts of trimethylamine were formed when choline was incubated with a liver homogenate. Bernheim (3) later demonstrated the presence of a specific enzyme, choline oxidase, in the liver. This enzyme is responsible for the oxidation of the alcohol group of the choline molecule.

II. Physiological Activity of Choline

The biologic activity of choline is quite complex, and much is still to be learned concerning its exact role in normal physiological function. It is apparent,

however, that the biological role of choline is divided into two major categories:

1. Its function as a lipotropic agent.
2. Its function as a source of labile methyl groups.

It appears that choline is essential for the normal metabolism of lipids in the liver. A deficiency of dietary choline causes fatty infiltration of the liver in most animals due to defective fat metabolism. This has been attributed to the role of the choline molecule in reactions involving phospholipid formation (13).

The other important biochemical function of choline is the ability of the molecule to furnish a labile methyl group for biochemical reactions. Examples of compounds which are acceptors of these methyl groups are: norepinephrine, nicotinamide, homocysteine, and catecholamines (O-methylation) (11). It has been shown that the choline molecule itself is not a methyl donor, but that it must first be oxidized to betaine by choline oxidase (10). However, since betaine is not a lipotropic agent, a decrease in choline oxidase activity could cause symptoms of choline deficiency as far as its role as a methyl donor is concerned. On the other hand, an increase in choline oxidase activity in the liver could cause symptoms of choline deficiency in that its lipotropic activity would be reduced.

The overall function of choline in the biologic system must be seen as an equilibrium between its lipotropic activity as a whole molecule and its function as a source of methyl groups in biochemical reactions.

III. Theories Concerning the Absorption and Excretion of Choline

One of the current problems concerning the metabolism of choline is the great variation in the appearance of choline in the urine following administration by different routes and to animals of differing physiological condition. Also in question are the reasons for the appearance of appreciable quantities of trimethylamine in the urine following oral administration of choline. Factors which have been found to modify the appearance of choline and trimethylamine in the urine following choline administration are:

1. Route of administration
2. Condition of intestinal flora
3. Condition of hepatic function

The question of absorption of choline and its excretion as such, or as its metabolite trimethylamine, may be resolved into two differing schools of thought:

1. Choline, given orally, is metabolized, to a certain extent to trimethylamine before absorption. The trimethylamine appearing in the urine is, therefore, a result of its production from choline by intestinal

microorganisms.

2. Choline is absorbed from the intestine unchanged. It is then metabolized to trimethylamine in the liver and subsequently excreted in the urine.

Norris and Benoit (15) noted that rats excreted large amounts of trimethylamine, but no choline, in the urine following oral administration of choline, but no trimethylamine was excreted following intraperitoneal injection of choline. On the basis of these findings, they concluded that the appearance of trimethylamine in the urine following oral administration of choline is the result of bacterial metabolism of choline before absorption. They also noted that orally administered trimethylamine could be recovered quantitatively from the urine in a 24 hour period. Dyer and Wood (12) demonstrated the ability of certain intestinal bacteria to transform choline to trimethylamine. De la Huerga and Popper (5) found substantially the same results as Norris and Benoit when they gave choline orally to humans. They noted that 60 to 67 per-cent of orally administered choline was excreted as trimethylamine in the urine in a 24 hour period. They also observed that prior administration of Aureomycin or Sulfathalidine orally greatly reduced the amount of trimethylamine excreted in the urine after oral choline due to inhibition of normal activity of the intestinal flora. When

they incubated choline with a dilution of human feces in a tryptose nutrient medium for 24 hours, they found that 30 to 40 percent of the choline had been metabolized to trimethylamine. With this information they concluded that substantial amounts of trimethylamine are produced by the action of intestinal bacteria on choline.

In a subsequent study (6) de la Huerga and Popper found that about 9 percent of intravenously administered choline was excreted unchanged in the urine by human subjects in 24 hours. During the same period no trimethylamine was excreted. They surmized that the absence of trimethylamine in the urine was due to the fact that the choline had not been exposed to intestinal bacteria.

In a series of experiments on rats, Popper (18) found that acute hepatic injury had no effect on trimethylamine excretion after oral doses of choline, but that chronic liver damage caused a decrease in trimethylamine excretion proportional to the degree of hepatic injury. He concluded that choline is metabolized in the intestine and that chronic hepatic damage alters trimethylamine excretion either by inhibiting the activity of the intestinal bacteria or by allowing a more rapid absorption of choline. This study also involved incubation of the contents of various levels of the rat intestinal tract with a tryptose media containing choline. It was

found that the bacteria in virtually every level of the intestine were able to convert choline to trimethylamine in appreciable amounts.

Pelner (17) found that normal patients and those with hepatitis excreted no choline within 24 hours after an oral dose of choline. Patients with cirrhosis, however excreted smaller amounts of trimethylamine and appreciable quantities of choline in the urine.

De la Huerga (8) gave choline orally to normal patients and recovered 50 to 60 percent of administered choline Nitrogen as trimethylamine Nitrogen from the urine in a 24 hour period. Following treatment with Aureomycin, Terramycin, and penicillin, the recovery of choline nitrogen as trimethylamine nitrogen was reduced to 12 to 16 percent within 24 hours. Oral treatment with Sulfathalidine, however, caused no change. In an earlier report (5), these same workers presented evidence that prior administration of Sulfathalidine significantly reduced trimethylamine excretion after choline ingestion.

In a study using germ-free rats, Prentiss (19) found an excretion rate of about 0.6 percent of an oral dose of choline as trimethylamine within 24 hours after the dose. This is in contrast to the 35 percent excretion of choline as trimethylamine observed in normal rats. Neither group of rats excreted choline after oral doses.

Both groups, however, quantitatively excreted oral doses of trimethylamine in the urine. On the basis of this evidence, they concluded that the appearance of trimethylamine in the urine is a result of choline metabolism by intestinal bacteria.

Although there is considerable evidence, summarized above, to suggest that choline metabolism in the intestinal tract is the primary source of urinary trimethylamine, there is about an equal amount of evidence to the contrary. Toda (23) and Artom and Crowder (1) established that liver slices and liver homogenates are capable of converting choline to trimethylamine in appreciable quantities.

Vetper and associates (25) instilled choline into ileal loops of dogs and were able to recover 30 to 40 percent of the instilled choline unchanged in the mesenteric venous blood in a period of 2 hours. This suggests that choline is readily absorbed from the intestine as such and is actually in contact with the intestinal flora for a relatively short time.

In a study using contents from several levels of the gastrointestinal tract, de la Huerga and Popper (7) incubated a tryptose medium containing choline with these contents for 24 hours. Although there was some conver-

sion of choline in most cases, the extent of metabolism had no connection with bacterial count.

Rhose and Searle (20) instilled choline into an isolated dog intestinal loop and incubated it. They found that only a very small amount of choline is converted to trimethylamine under these conditions, and concluded that no important loss of choline takes place through the action of intestinal microorganisms.

In a series of experiments using rats, Riedesel and Hines (21) found that about 40 percent of choline instilled into the small intestine disappears in a 4 hour period. After almost complete inhibition of the intestinal flora with Aureomycin and sulfaguanidine there was no observed change in the rate of choline disappearance. Also, incubation of choline with an intestinal homogenate and a balanced saline solution for 2 hours produced no detectable quantities of trimethylamine.

IV. Investigations Undertaken in This Study

Due to the long-standing and as yet unresolved question dealing with the relative mertis of the path by which choline leaves the intestine, these studies were carried out in an attempt to resolve some of the obvious contradictions apparent in the literature. The following is a brief listing of the course of experimentation:

1. A study of the rate of trimethylamine excretion in the rabbit following oral administration of choline chloride.

2. A study of the rate of trimethylamine formation while incubating choline chloride with the intestinal contents of rabbits, in an attempt to correlate the rate of urinary excretion of trimethylamine with the rate of its production from choline by intestinal bacteria.

3. A study of the rate of trimethylamine formation when choline is incubated with rat intestinal content using different incubation media and incubation periods. This was done in an attempt to determine whether the rate of trimethylamine formation *in vitro* may be influenced by the period of incubation and the incubation employed.

EXPERIMENTAL PROCEDURE

I. Materials

All chemical reagents used in these investigations are listed in the appendix.

All animals used were purchased from a licensed animal supplier and maintained on a diet of Purina Laboratory Chow and water. The rabbits were male domestics weighing an average of 2.5 kilograms. The rats used were males of the Wistar strain weighing between 140 and 160 grams. Prior to any experiments, the animals were fasted for 12 hours, but water was allowed ad libitum.

II. Methods

For an excretion study, the rabbit was lightly anesthetized with chloroform, and a solution of choline chloride was administered intragastrically via a catheter and syringe. It was found necessary to lightly anesthetize the animals during this procedure to facilitate intubation. The solution of choline was administered slowly to prevent damage to the stomach walls. Following choline administration, a small amount of water was added to the syringe and the solution remaining in the catheter flushed into the stomach. The animal was then catheterized using a 10 FR Foley Catheter having a 3 cc inflatable bulb. After insertion, the bulb was inflated by adding 3 cc of air from a syringe through a needle.

The animal was then placed in a specially constructed box which allowed it to remain relatively comfortable but immobilized for the duration of the study. The animal was given water periodically during the collection of urine, but food was witheld. The urine was collected in an erlenmeyer flask containing 5 cc of 50 percent hydrochloric acid.

At the end of the collection period, the urine was alkalinized to pH 10 with sodium hydroxide. Activated charcoal was added to the urine (2 grams per 50 cc urine), the mixture shaken, and allowed to stand for 1 hour. This mixture was then filtered through a double thickness of Whatman No. 24 filter paper using suction. This method, as described by de la Huerga and Popper (5), eliminates all insoluble material and produces an almost colorless filtrate. The pH was then determined again because the urine, in a few cases, became acidic during the above process. If necessary, more sodium hydroxide was added.

The alkaline pH was selected because Bandelin and Pankratz (2) showed that choline can be precipitated as the reineckate from a solution at pH 10, whereas trimethylamine remains in solution.

The amount of choline in the solution was determined by the gravimetric method of Pankratz and Bandelin (16). A volume of 3 percent ammonium reineckate in anhydrous

methanol was added, the amount equal to one-half the volume of urine filtrate to be analyzed. In the analyses where choline was present, the method of addition of reineckate was of importance. If the reineckate was added slowly with a minimum of agitation, large crystals were formed which filtered easily. However, rapid addition and excessive agitation produced very small crystals which clogged the sintered glass filters and made further filtration almost impossible. The solutions containing the precipitates were placed in the refrigerator for 4 hours to allow maximum precipitation (21).

Fine sintered glass filters and vacuum were used to collect the precipitate. If, during the filtration procedure, the precipitate became too dry, the sintered glass tended to clog and interfere with filtration. Thus, careful timing of the several steps was found to facilitate complete filtration and washing. The precipitate was washed with several portions of distilled water and finally dried by suction. The filter crucibles were then placed in the oven at 100 degrees centigrade for 1 hour, cooled in a dessicator, and weighed.

The difference between the weight of the crucible plus the precipitate and the weight of the crucible after dissolving the choline reineckate with acetone gave the

weight of choline reineckate. It was not possible to determine the weight of the choline reineckate by subtracting the weight of the tared crucible from the weight of the crucible containing the precipitate because small quantities of acetone insoluble precipitates were formed by the addition of the reineckate solution. If the tare weight of the crucible had been used, the analysis would have resulted in falsely high values for the amount of choline reineckate. The weight of choline reineckate was multiplied by 0.3304 to determine the equivalent value of choline chloride (16).

Following filtration of the choline reineckate precipitate, the remaining filtrate was acidified to pH 2 by addition of hydrochloric acid as determined with pHydrion Paper. Upon acidification of the filtrate, a precipitate of trimethylamine reineckate separated immediately without addition of further reineckate. The same gravimetric process was used as described before to determine the weight of trimethylamine reineckate. The weight of trimethylamine reineckate obtained was multiplied by 0.2349 (21) to obtain the equivalent weight of trimethylamine.

Investigation of the Ability of Intestinal Bacteria To Metabolize Choline Chloride

The rabbits used in this portion of the study were quickly killed with chloroform, a midline incision made,

and ligatures applied to the intestines at the pylorus and just above the caecum. The ligated portion of the intestine was removed and its contents flushed into and erlenmeyer flask with the chosen media. To this mixture was added an amount of choline chloride equivalent to that administered in the excretion studies. The contents of the flasks were mixed thoroughly and placed in an incubator at 37 degrees Centigrade for 24 hours.

The rats used in this study were killed with chloroform, and their intestinal contents removed in a manner similar to that described above. Choline chloride was added to the mixture of intestinal contents and medium, mixed thoroughly, and placed in an incubator at 37 degrees Centigrade for 24 hours.

In order to determine the choline and trimethylamine in the incubated mixtures, it was first necessary to precipitate the proteins in the mixtures. The protein present was from 2 sources; the Lauryl Tryptose Broth and the intestinal fluid. The method used to precipitate the proteins was the method of Somogyi (22) as modified by Riedesel (21), described as follows:

A 0.3 N barium hydroxide solution was added to the contents of the erlenmeyer flask. In all cases it was necessary to add greater amounts to the flasks containing

the Lauryl Tryptose Broth due to the presence of proteins in the medium itself. Five drops of phenolphthalein indicator solution were added, after which the contents of the erlenmeyer flask were treated with a 0.3 N solution of zinc sulfate until the pink color disappeared. If the supernatant fluid cleared rapidly at this time, additional barium hydroxide solution was added to precipitate the excess zinc sulfate and turn the solution pink. Complete precipitation of proteins was indicated when the supernatant liquid remained clear, and this occasionally required empirical judgment as to total quantities of precipitant.

The pH of the solution was adjusted to 10 using sodium hydroxide, testing with pHydrion paper. The final determinations of choline and trimethylamine were then conducted as previously described.

III. Evaluation of Methods

Recovery of Choline Chloride from Urine

In order to evaluate the accuracy of the gravimetric method employed to analyze the choline content of urine, a series of control studies was performed. Several male domestic rabbits were fasted for 12 hours, and their urine was collected for 24 hours. No food was allowed during the collection period. The urine was collected in the same manner as in the trimethylamine excretion experiment. Analysis of the urine samples indicated that no choline or trimethylamine was present. A series of 20 milliliter samples was used. Varying amounts of choline chloride were added to these samples, and an attempt was made to quantitatively recover this from the samples. The gravimetric method previously described was used in these analyses. The results of these evaluations (Table I) indicate that the method employed for the determination of choline chloride in urine samples is of sufficient accuracy for these experiments.

Recovery of Trimethylamine from Urine

The evaluation of the accuracy of the gravimetric method for determination of trimethylamine in urine was conducted in much the same way as that for choline above. Crystalline trimethylamine hydrochloride was obtained and

Table I

Experiment to Evaluate the Accuracy of the Gravimetric Method Used in Determinations of Choline Chloride in Urine Samples.

Sample number	Milligrams choline chloride added	Milligrams choline chloride recovered	Percent Recovered
1	0	0	
2	0	0	
3	20	19.43	97.2
4	20	19.86	99.3
5	30	29.38	98.0
6	30	30.62	102.0
7	40	40.80	102.0
8	40	40.56	101.4
9	50	50.07	100.1
10	50	51.21	102.4
11	60	58.70	97.8
12	60	59.82	99.7
13	70	68.14	97.3
14	70	71.63	102.3

This table represents the accuracy of the gravimetric method in recovery of known quantities of choline chloride from urine samples. Mean = 99.9 percent . Standard Deviation = 1.79

purified as described in the appendix. The molecular weight of trimethylamine hydrochloride is 96.6 and that of trimethylamine base is 59.1. Since trimethylamine base was not available, samples of trimethylamine hydrochloride equivalent to a given amount of trimethylamine base were used. A stock solution containing 164 milligrams of trimethylamine hydrochloride per milliliter was prepared. This solution is equivalent to 100 milligrams of trimethylamine base per milliliter of solution. This allowed convenient measurement of the desired amounts of trimethylamine. The control urine samples used in this test were found to be free of any choline or trimethylamine. Measured quantities of trimethylamine were added to the urine samples and analysis was done using the gravimetric method. The results of this study are given in Table II. It may be seen that this gravimetric method is of sufficient accuracy for these experiments.

Table II

Evaluation of the Gravimetric Method in Determinations of Trimethylamine in Urine Samples.

Sample Number	Milligrams trimethylamine added	Milligrams trimethylamine recovered	Percent recovered
1	0	0	
2	10	9.86	98.6
3	20	20.73	103.6
4	30	30.92	103
5	40	38.75	96.9
6	50	50.71	101.4
7	60	61.24	102.7
8	70	72.08	102.9
9	80	79.40	99.2
10	90	91.98	102.2
11	100	99.03	99.0

This table represents the accuracy of the gravimetric method in recovery of known quantities of trimethylamine from urine samples. Mean = 100.9 per cent, Standard Deviation = 2.12.

Recovery of Choline Chloride and Trimethylamine From Urine.

The following procedures were done to determine the accuracy of the gravimetric method employed here to separate and quantitatively recover choline and trimethylamine from urine samples containing both substances. Urine was collected from fasted male rabbits and was found to contain neither choline nor trimethylamine. Measured amounts of choline and trimethylamine were added to these samples and an attempt made to recover quantitatively each substance by the gravimetric method. As indicated in Table III, recovery of the trimethylamine was, in most instances, incomplete. This may have been due to precipitation of some trimethylamine along with choline when reineckate was added at an alkaline pH. However, it was decided that this method is sufficiently accurate since only relative values of choline and trimethylamine were sought here. If absolute values with a very high degree of accuracy were desired, some modification of the method would be indicated.

Table III

Results of trial Quantitative Separation and Recovery of Choline Chloride and Trimethylamine from Urine Samples.

Sample number	C	CR	%C	T	TR	%T
1	0	0		10	9.81	98.1
2	10	9.97	99.7	0	0	
3	20	20.31	101.5	10	9.59	95.9
4	10	10.44	104.4	20	19.06	95.3
5	10	10.04	100.4	10	9.63	96.3
6	30	30.88	102.9	10	9.79	97.9
7	30	29.82	99.4	20	19.11	95.6
8	30	30.60	102.0	30	28.72	95.7
9	40	39.44	96.6	30	29.40	98.0
10	50	51.03	102.1	40	38.96	97.3
11	60	60.81	101.3	50	48.62	97.2
12	70	68.09	97.3	60	58.47	97.5
13	70	71.02	101.5	70	69.36	99.1
14	80	82.70	103.4	80	82.19	102.7

Key: C = Milligrams choline chloride added
CR = Milligrams choline chloride recovered
%C = Per-cent choline chloride recovered
T = Milligrams trimethylamine added
TR = Milligrams trimethylamine recovered
%T = Per-cent trimethylamine recovered

This table represents the accuracy of the gravimetric method in the recovery of choline and trimethylamine from urine.
Mean %C = 100.9, Standard Deviation = 2.9
Mean %T = 97.6, Standard Deviation = 1.93

Recovery of Trimethylamine from Intestinal Contents

A. Rabbits

In order to evaluate the accuracy of the gravimetric method to quantitatively recover trimethylamine from intestinal contents, two male rabbits were fasted for 12 hours. The intestinal contents were collected as previously described, using Dulbecco's Phosphate Buffered Saline solution to flush the contents from one rabbit, and Lauryl Tryptose Broth for the other. Several 20 milliliter samples were removed from each flask, a measured amount of trimethylamine added to each, and the proteins immediately precipitated with barium hydroxide and zinc sulfate. The samples were then analyzed for trimethylamine using the gravimetric method.

B. Rats

The intestinal contents of several rats were collected and prepared as described under Rabbits above. Dulbecco's Phosphate Buffered Saline solution was used to flush the contents from six rats, and Lauryl Tryptose Broth for six other animals. A measured amount of trimethylamine was added to each sample, after which the proteins were precipitated. Each sample was then analyzed for trimethylamine using the gravimetric method.

The results of these tests, summarized in Tables IVa and IVb, indicate that the gravimetric method was sufficiently accurate for the purpose of this study in analyzing intestinal contents for trimethylamine.

TABLE IVa

Test of the Gravimetric Method in Quantitative Recovery of Trimethylamine from Rabbit Intestinal Contents.

Sample number	Media used*	Milligrams trimethylamine added	Milligrams trimethylamine recovered	Percent trimethylamine recovered
1	T	0	0	
2	T	20	19.34	96.7
3	T	20	20.71	103.5
4	T	40	39.47	98.7
5	T	40	38.97	97.4
6	T	60	60.82	101.3
7	D	0	0	
8	D	20	20.63	103.1
9	D	20	20.85	104.2
10	D	40	38.72	96.8
11	D	40	41.61	104.0
12	D	60	61.09	101.8

* T = Lauryl Tryptose Broth
  D = Dulbecco's Phosphate Buffered Saline

This table represents the accuracy of the gravimetric method in the recovery of trimethylamine from intestinal contents.
Mean = 100.6 percent
Standard Deviation = 2.55

Table IVb

Test of the Gravimetric Method in Quantitative Recovery of Trimethylamine from Rat Intestinal Contents.

Sample number	Media used*	Milligrams trimethylamine added	Milligrams trimethylamine recovered	Percent trimethylamine recovered
1	T	0	0	
2	T	20	19.82	99.1
3	T	20	19.47	97.3
4	T	40	38.84	97.0
5	T	40	41.03	102.5
6	D	0	0	
7	D	20	20.55	102.7
8	D	20	19.48	97.4
9	D	40	41.27	103.2
10	D	40	40.34	100.9

* T = Lauryl Tryptose Broth
  D = Dulbecco's Phosphate Buffered Saline

This table represents the accuracy of the gravimetric method in the recovery of trimethylamine from rat intestinal contents.
Mean = 100.0 percent
Standard Deviation = 2.20

Comparison of Tables IV a and IVb indicate that trimethylamine may be recovered with comparable accuracy from either rat or rabbit intestinal contents.

IV. Rate of Urinary Trimethylamine Excretion Following Oral Administration of Choline Chloride

A series of preliminary studies was made to determine if choline or trimethylamine are present in the urine of normal rabbits. Since the rate of trimethylamine and choline excretion was to be determined after administration of choline, any endogenous source of excretion would seriously interfere with the experimental results. It was found that animals which had been allowed access to food until the time of urine collection excreted substances in the urine which produced precipitates on addition of ammonium reineckate. However, animals which had been fasted for a period of 12 hours prior to urine collection excreted urine which formed no precipitate on addition of reineckate. It was therefore reasoned that any choline or trimethylamine found in the urine after choline administration would be a result of the metabolism and excretion of the choline. As an extra matter of control, the urine collected immediately after catheterization was analyzed for choline and trimethylamine for each animal. This urine was free of choline and trimethylamine in all animals except two, and these were rejected.

Since all rabbits used weighed between 2.3 and 2.7 kilograms, a standard dose of 2.0 grams of choline chloride was decided on. This is equivalent to approximately

800 milligrams of choline chloride per kilogram of body weight. This amount of choline chloride is equivalent to 200.06 milligrams of nitrogen, and subsequent calculations of per-cent nitrogen excretion were simplified.

The rabbits were fasted for 12 hours, after which each received the 2.0 grams of choline chloride. Urine was collected for 24 hours. The urine samples were analyzed for trimethylamine, and the results of these analyses are summarized in table V.

Table V

Amounts of Trimethylamine Excreted in a 24 Hour Period Following Oral Administration of 2.0 Grams of Choline Chloride*

Animal number	Milligrams trimethylamine recovered	Milligrams trimethylamine Nitrogen recovered	Percent choline Nitrogen excreted as trimethylamine Nitrogen
1	371.70	88.09	44.0
2	368.94	87.44	43.7
3	360.47	85.43	42.7
4	355.85	85.35	42.2
5	349.73	82.79	41.4
6	342.78	81.24	40.6
7	324.18	76.84	38.4
8	309.71	73.39	36.7
9	309.50	73.35	36.6
10	296.48	70.27	35.1
11	291.77	69.13	34.5
12	287.60	67.9	34.1
13	279.09	66.14	33.1
14	253.40	60.1	30.1
15	241.06	56.5	28.5

Mean per-cent of choline Nitrogen excreted as trimethylamine Nitrogen in 23 hours = 37.4 (Standard Deviation = 4.62).

* Equivalent to 200.06 mg nitrogen

V. Production of Trimethylamine from Choline by Intestinal Bacteria

If the trimethylamine excreted in the urine after oral administration of choline is a result of the action of intestinal bacteria on the choline, it was reasoned that *in vitro* incubation of choline with intestinal bacteria and a suitable mediumwould produce amounts of trimethylamine comparable to those presented in Table V.

Rhose (20) and Riedesel (21) have found no trimethylamine production after incubation of choline with intestinal contents. However, de la Huerge (7) and Dyer (12) reported significant trimethylamine formation after incubation of choline with intestinal contents. Riedesel used a balanced saline solution as the incubation medium. Dyer and de la Huerga used a tryptose nutrient broth in their incubation studies.

A. Rabbit Intestinal Content incubated with Dulbecco's Solution

Male rabbits were fasted for 12 hours, killed quickly with chloroform after which their intestinal content was removed by flushing with Dulbecco's Solution into an erlenmeyer flask. To each flask was added the two grams of choline, after which they were incubated at 37 degrees Centigrade for 24 hours.

Following incubation the mixtures were analyzed for trimethylamine. Three of the samples were found to be free of trimethylamine. The results are summarized in Table VI.

B. Rabbit Intestinal Contents Incubated with Lauryl Tryptose Broth

Lauryl Tryptose Broth is recommended as an excellent mediumfor the growth and metabolism of coliform bacteria (9). It was thought that this medium would provide ideal conditions for the metabolism of choline by intestinal microorganisms.

The intestinal content of the rabbits was collected as described above using Lauryl Tryptose Broth as the flushing agent. The two grams of choline chloride was added to each sample, and the mixtures incubated at 37 degrees Centigrade for 24 hours. Trimethylamine was again determined as previously described. The results of these analyses are presented in Table VII. Trimethylamine was recovered from each of these samples.

C. Rat intestinal Content

Prentiss (19) determined that an average of 35 per-cent of orally administered choline nitrogen is excreted in the urine as trimethylamine nitrogen in a 24 hour period by normal rats. Riedesel (21), as mentioned previously, found no trimethylamine production on incubation of choline with rat intestinal contents for 2 hours.

Table VI

Amount of Trimethylamine Formed on Incubation of 2.0 grams of Choline Chloride (Equivalent to 200.06 Milligrams Nitrogen) with Rabbit Intestinal Contents Using Dulbecco's Phosphate Buffered Saline as the Incubation Media at 37 Degrees Centigrade for 24 hours.

Animal Number	Milligrams trimethylamine recovered	Percent choline Nitrogen recovered as trimethylamine Nitrogen
1	26.14	3.1
2	25.70	3.05
3	20.61	2.4
4	12.08	1.4
5	0	0
6	0	0
7	0	0

Mean percent choline Nitrogen recovered as trimethylamine Nitrogen in 24 hours = 1.42 (Standard Deviation = 1.34)

Table VII

Amount of Trimethylamine Formed on Incubation of 2.0 grams of Choline Chloride (Equivalent to 200.06 Milligrams Nitrogen) with Rabbit Intestinal Contents Using Lauryl Tryptose Broth as the Incubation Media at 37 Degrees Centigrade for 24 Hours.

Animal number	Milligrams trimethylamine recovered	Percent choline Nitrogen recovered as trimethylamine Nitrogen
1	291.52	34.5
2	266.35	31.5
3	262.02	31.1
4	221.43	26.2
5	201.51	23.9
6	157.77	18.7
7	140.67	16.7

Mean percent choline Nitrogen recovered as trimethylamine Nitrogen in 24 hours = 26.0 (Standard Deviation = 6.24)

De la Huerga (7), using a tryptose nutrient medium and rat intestinal contents found an average of about 40 percent of choline nitrogen recoverable as trimethylamine nitrogen after a 24 hour incubation period. In an attempt to resolve these conflicting reports, incubation studies using rat intestinal content with choline were conducted under several different conditions:

1. Dulbecco's Phosphate Buffered Saline and a 24 hour incubation period.

2. Dulbecco's Phosphate Buffered Saline and a 2 hour incubation period.

3. Lauryl Tryptose Broth and a 24 hour incubation period.

4. Lauryl Tryptose Broth and a 2 hour incubation period.

The intestinal content of the rats was flushed into erlenmeyer flasks, using the appropriate medium. To each flask was added 100 milligrams of choline chloride (equivalent to 10 milligrams nitrogen). Incubation at 37 degrees Centigrade was performed for 2 and 24 hours. The samples were then analyzed for trimethylamine. The results of incubation of rat intestinal contents with choline and Dulbecco's Phosphate Buffered Saline are summarized in Table VIII. The results of the

incubation of choline chloride with rat intestinal contents and Lauryl Tryptose Broth are presented in Table IX.

Table VIII

Amount of Trimethylamine Produced by Incubation of 100 Milligrams of Choline Chloride with Rat Intestinal Contents and Dulbecco's Phosphate Buffered Saline at 37 Degrees Centigrade for Periods of 2 and 24 hours.

Animal number	Incubation period (hours)	Milligrams Trimethylamine recovered	Percent choline Nitrogen recovered as trimethylamine Nitrogen
1	2	1.39	2.2
2	2	0	0
3	2	0	0
4	2	0	0
5	24	4.09	9.6
6	24	3.22	7.6
7	24	2.40	5.7
8	24	2.01	4.9
9	24	1.96	4.6

Mean percent choline Nitrogen recovered as trimethylamine Nitrogen in 2 hour incubation period = 0.52 (Standard Deviation = 0.95).

Mean percent choline Nitrogen recovered as trimethylamine Nitrogen in 24 hour incubation period = 6.5 (Standard Deviation = 1.92).

Table IX

Amount of Trimethylamine Produced by Incubation of 100 Milligrams of Choline Chloride with Rat Intestinal Contents and Lauryl Tryptose Broth at 37 Degrees Centigrade for Periods of 2 and 24 hours.

Animal number	Incubation period (hours)	Milligrams trimethylamine recovered	Percent choline Nitrogen recovered as trimethylamine Nitrogen
1	2	1.80	4.2
2	2	0.77	1.8
3	2	0	0
4	2	0	0
5	24	16.41	37.9
6	24	13.01	30.8
7	24	12.60	29.8
8	24	11.84	28.1
9	24	10.87	25.8

Mean percent choline Nitrogen recovered as trimethylamine Nitrogen in 2 hour incubation period = 1.5 (Standard Deviation = 1.79).

Mean percent choline Nitrogen recovered as trimethylamine Nitrogen in 24 hour incubation period = 30.7 (Standard Deviation = 4.33).

DISCUSSION

Following oral administration of choline, considerable amounts of trimethylamine are excreted in the urine. Norris and Benoit (15), Prentiss(19), and Popper (18) found that normal rats excrete from 35 to 40 percent of orally administered choline as trimethylamine in a 24 hour period. De la Huerga and Popper (5) determined that humans excrete about 60 percent of an oral dose of choline as trimethylamine. Inhibition of the intestinal flora with antimicrobial agents was found to substantially reduce the excretion of trimethylamine under the same conditions (5). Germ-free rats excrete virtually no trimethylamine after oral administration of choline (19). When choline is incubated with intestinal bacteria using a nutrient medium considerable amounts of trimethylamine are produced (12, 5). Parenteral administration of choline, however, results in virtually no excretion of trimethylamine (6, 15, 17). These findings have led to the belief that urinary excretion of trimethylamine after oral administration of choline is the result of conversion of choline to trimethylamine by intestinal bacteria before absorption.

Toda (23) and Artom and Crowder (1) determined that liver slices and liver homogenates are capable of

converting choline to trimethylamine in appreciable quantities. Vetper (25) found that about 40 percent of choline instilled into intestinal loops of dogs could be recovered unchanged from the mesenteric venous blood in a 2 hour period. Rhose and Searle (20) reported no trimethylamine formation after incubation of an isolated dog ileal loop containing choline for 2 hours. Riedesel (21) found that choline rapidly disappears from the rat intestine. However, he was unable to demonstrate trimethylamine formation when an intestinal homogenate was incubated with choline in a balanced saline solution. It would appear from these findings that choline is rapidly absorbed from the small intestine, and that it is in contact with the intestinal microorganisms for a relatively short time. From this information, it is theorized that choline is absorbed unchanged from the intestine and is metabolized to trimethylamine by liver enzymes.

The present studies show that rabbits excrete an average of 37.4 percent of orally administered choline as trimethylamine in 24 hours (Table V). Incubation of choline with rabbit intestinal contents using Dulbecco's Phosphate Buffered Saline as the incubation medium for 24 hours results in a mean of 1.42 percent of the choline converted to trimethylamine (Table VI). The same

procedure using Lauryl Tryptose Broth as the incubation medium results in an average of 26.0 percent conversion of choline to trimethylamine (Table VII). It may be seen that insufficient amounts of trimethylamine are produced by intestinal bacteria, even in the presence of an ideal growth medium to account for the amount of trimethylamine excreted in the urine after oral administration of choline. In addition, insignificant quantities of trimethylamine are produced when a balanced saline solution is used as the incubation medium. It is therefore reasoned that the appearance of trimethylamine in the urine after after oral administration of choline cannot be exclusively attributed to the bacterial metabolism of choline to trimethylamine in the intestine. This process is undoubtedly a major factor in choline metabolism, but other pathways must be involved. It is likely that metabolism of choline by liver enzymes is a contributing factor in the appearance of trimethylamine in the urine.

The conflicting reports concerning the ability of intestinal bacteria to convert choline to trimethylamine <u>in vitro</u> seems to be due to differences in experimental method. Riedesel (21) used a balanced saline solution as the incubation medium when incubating rat intestinal content with choline. He reported no

trimethylamine production in a 2 hour period. De la Huerga (5), using a tryptose nutrient medium, found that intestinal bacteria are capable of converting appreciable quantities of choline to trimethylamine within 24 hours. The differences in experimental method may be seen to be incubation period and the incubation medium employed.

The studies presented here indicate that very little trimethylamine is produced from choline by intestinal bacteria in 2 hours using either a balanced saline solution or a tryptose nutrient broth as the incubation medium. (Tables VIII and IX). A 24 hour period of incubation caused production of trimethylamine with both the balanced saline solution and the nutrient broth. This evidence indicates that the major factor in trimethylamine production from choline <u>in vitro</u> is the incubation period employed. An average of 6.5 percent of choline was converted to trimethylamine when incubated with rat intestinal contents for 24 hours using Dulbecco's solution as the incubation medium (Table VIII), whereas a mean conversion percentage of 30.7 was observed under the same conditions using Lauryl Tryptose Broth (Table IX). Statistical analysis shows a significant difference at the 0.98 level. Incubation of choline and rabbit intestinal content for 24 hours using Dulbecco's as the incubation medium resulted in insignificant production of trimethylamine (mean = 1.42 percent). When the Lauryl Tryptose Broth

was used in place of Dulbecco's solution in the same procedure, however, appreciable quantities of trimethylamine were recovered (mean = 26.0 percent). The means were found to be significantly different at the 0.98 level.

These findings indicate that the amount of trimethylamine produced by the action of intestinal bacteria on choline *in vitro* is greatly influenced by the type of incubation medium used and the incubation period.

SUMMARY

The urine of rabbits was analyzed for trimethylamine after oral administration of choline. It was found that an average of 37.4 percent of an oral dose of choline was excreted in the urine as trimethylamine in a 24 hour period. Incubation of choline with rabbit intestinal content for 24 hours using a balanced saline solution as the incubation medium resulted in an average of 1.42 percent conversion of choline to trimethylamine. The same procedure using a tryptose broth as the incubation medium resulted in a mean of 26,0 percent conversion of choline to trimethylamine. It was concluded that appearance of trimethylamine in the urine following oral administration of choline cannot be attributed exclusively to metabolism of choline by intestinal microorganisms.

Incubation of rat intestinal content with choline using a nutrient tryptose broth or a balanced saline solution resulted in insignificant formation of trimethylamine in 2 hours. Incubation using a tryptose nutrient medium for 24 hours caused an average of 30.7 percent conversion of choline to trimethylamine. Using a balanced saline solution under the same conditions resulted in an average of 6.5 percent conversion of choline to trimethylamine. It was concluded that the

amount of trimethylamine produced by the action of intestinal bacteria on choline *in vitro* is greatly influenced by the type of incubation medium employed and the incubation period.

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APPENDIX

Appendix

Chemical Reagents

Activated Charcoal U.S.P.

Ammonium Reineckate. Ammonium Reineckate 3 percent solution in anhydrous methanol. All solutions were prepared freshly on the day of use and filtered by gravity.

Barium Hydroxide. Barium hydroxide 0.3 N solution in distilled water.

Choline Chloride. The choline chloride was dissolved in absolute ethyl alcohol, cooled, and precipitated by the addition of cold ether. The crystals were collected on sintered glass filters, dried at 100 degrees Centigrade for 4 hours, and stored in a desiccator over anhydrous calcium chloride. All choline chloride solutions were prepared freshly on the day of use in distilled water.

Dulbecco's Phosphate Buffered Saline (14)

material	grams/liter
NaCl	8.0
KCl	0.2
Na_2HPO_4	1.15
KH_2PO_4	0.2
$CaCl_2$	0.1
$MgCl_2.6H_2O$	0.1

Hydrochloric Acid. Hydrochloric Acid U.S.P., 50 percent in distilled water.

Lauryl Tryptose Broth (Difco Co.) (9)

material	grams/liter
Bacto-Tryptose	20
Bacto-Lactose	5
K_2HPO_4	2.75
KH_2PO_4	2.75
NaCl	5
Sodium Lauryl sulfate	0.1

Phenolphthalein Indicator Solution.

Sodium Hydroxide. Sodium Hydroxide U.S.P., 50 percent in distilled water.

Trimethylamine Hydrochloride. The trimethylamine hydrochloride was dissolved in anhydrous ethyl alcohol, cooled, and precipitated by the addition of cold ether. The crystals were collected in fine sintered glass filters, dried at 100 degrees centigrade for 4 hours, and stored in a dessicator over anhydrous calcium chloride.

Zinc Sulfate. Zinc Sulfate, 0.3N solution in distilled water.